Phosphorylation of the *Nicotiana benthamiana* WRKY8 Transcription Factor by MAPK Functions in the Defense Response

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INTRODUCTION

Plants have evolved a system of defense responses to protect themselves against colonization by pathogens. The plant innate immunity system consists of two primary layers. One layer relies on the perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (Zipfel, 2008). This recognition activates basal defense to prevent penetration and to restrict growth of pathogens (Jones and Dangl, 2006). The second layer is the recognition of pathogen effector molecules through host resistance (R) proteins. R gene–mediated resistance triggers strong gene-for-gene resistance that often includes hypersensitive response (HR) cell death.

Mitogen-activated protein kinase (MAPK) signaling pathways have important roles in basal defense and R gene–mediated resistance. Many studies have characterized the tobacco (*Nicotiana tabacum*) MAPKs, wound-induced protein kinase (WIPK; Seo et al., 1995), and salicylic acid–induced protein kinase (SIPK; Zhang and Klessig, 1997) and their orthologs in other plant species (Katou et al., 2005; Nakagami et al., 2005). WIPK and SIPK participate either in N gene–dependent resistance against Tobacco mosaic virus (Zhang and Klessig, 1998; Jin et al., 2003), Cf-9–dependent responses to *Cladosporium fulvum*–derived elicitor *Avr9* (Romeis et al., 1999), or in PAMP-mediated basal defense against the fungal pathogen *Colletotrichum orbiculare* (Tanaka et al., 2009). WIPK and SIPK share MEK2, a common upstream mitogen-activated protein kinase kinase (MAPKK; Yang et al., 2001). Expression of MEK2DD, a constitutively active mutant of MEK2, induces HR-like cell death, defense gene expression, and generation of nitric oxide and reactive oxygen species, all preceded by activation of endogenous WIPK and SIPK (Yang et al., 2001; Ren et al., 2002; Yoshioka et al., 2003; Asai et al., 2008). Transgenic potato (*Solanum tuberosum*) plants carrying MEK2DD driven by a pathogen-inducible promoter show resistance to *Phytophthora infestans* and *Alternaria solani* (Yamamizo et al., 2006). MPK3 and MPK6 (*WIPK* and SIPK orthologs, respectively, in *Arabidopsis thaliana*) cooperatively regulate the production of the antifungal compound camalexin, an indolic phytoalexin of *Arabidopsis* (Ren et al., 2008). NTF4, which shares 93.6% identity with SIPK, is thought to be functionally redundant with SIPK in the defense-related signaling pathway (Ren et al., 2006; Asai et al., 2008). The cytokinesis-related MAPK cascade NPK1-MEK1-NTF6 is also essential for *N*– and *Pto*–dependent resistance (Jin et al., 2002; del Pozo et al., 2004; Liu et al., 2004), as well as for reactive oxygen species production (Asai et al., 2008). Despite extensive accumulation of evidence that shows the importance of MAPKs in basal defense
and R gene–mediated resistance, the mechanisms through which MAPKs transduce the signals are largely unknown.

WRKY proteins comprise a large family of plant transcription factors (Eulgem et al., 2000). WRKY family members are divided into three groups based on the number of WRKY domains and certain features of the zinc finger–like motifs (Eulgem et al., 2000). WRKY proteins with two WRKY domains belong to group I, and proteins with one WRKY domain belong to group II or III based on features of the zinc finger motif. WRKY proteins bind W-box sequences (TTGACC/T) in the promoter region of target genes (Eulgem et al., 2000; Ciolkowski et al., 2008). Loss-of-function and gain-of-function studies showed that WRKY transcription factors participate in defense responses either as positive or as negative regulators (Eulgem and Somssich, 2007; Shen et al., 2007). Some WRKYs are regulated by MAPKs at the transcriptional and posttranscriptional levels in defense-related signaling pathways. Arabidopsis WRKY22 and WRKY29 are transcriptionally induced by MPK3 and MPK6 and confer resistance to both bacterial and fungal pathogens (Asai et al., 2002). WRKYs are phosphorylated by MAPKs in vitro (Andreason et al., 2005; Katou et al., 2005; Menke et al., 2005; Popescu et al., 2009). However, whether these WRKYs are directly phosphorylated by MAPKs in vivo is unknown. Likewise, the effect of MAPK-mediated phosphorylation on the function of WRKY remains to be defined.

Here, we report that Nicotiana benthamiana WRKY8 is a physiological substrate of SIPK, NTF4, and WIPK. SIPK phosphorylates the SP cluster of WRKY8, which is highly conserved in the N-terminal region of group I WRKY proteins. SIPK-mediated phosphorylation of WRKY8 increased its binding activity to the W-box sequence and transactivation activity. Phospho-mimicking mutant and virus-induced gene silencing (VIGS) of WRKY8 showed that MAPK-mediated phosphorylation of WRKY8 has an important role in plant immunity through activation of downstream genes.

RESULTS

Multisite Phosphorylation of WRKY8 by SIPK

We previously identified proteins phosphorylated in vitro by St MPK1, an SIPK ortholog in potato, using an in vitro expression cloning method (Katou et al., 2005). In this study, we designated a clone that contains two putative WRKY domains as St WRKY8. N. benthamiana, like potato, belongs to Solanaceae and is useful to study biological functions by Agrobacterium tumefaciens–mediated transient expression and VIGS. We isolated a cDNA fragment of Nb WRKY8 from N. benthamiana by PCR using primers designed based on the St WRKY8 sequence. The deduced amino acid sequence of Nb WRKY8 contained two WRKY domains and belonged to group I WRKY proteins (see Supplemental Figure 1A online). Ser or Thr followed by Pro (SP or TP) is a minimal consensus motif for MAPK phosphorylation (Sharrock et al., 2000). WRKY8 contains seven potential MAPK phosphorylation sites, five of which are concentrated in the N-terminal region (SP cluster). Interestingly, WRKY8 also contained a deduced D domain adjacent to the N-terminal side of the SP cluster (see Supplemental Figure 1A online). The ability of MAPKs to effectively and specifically recognize their substrates is increased by their capacity to bind to those substrates with relatively high affinity. Often, this direct interaction occurs by way of MAPK-docking sites on substrate proteins (Sharrock et al., 2000). The D domain is the best described docking motif whose general features include a cluster of basic residues upstream of the LxL motif ([K/R]1-x2-6-[L/I]-x-[L/I]) (Sharrock et al., 2000). A closer examination of Arabidopsis, rice (Oryza sativa), and Solanaceae plant group I WRKY amino acid sequences showed that the SP cluster and deduced D domain are conserved in the N-terminal regions of several group I WRKYS, inferring a functional importance (see Supplemental Figure 1B and Supplemental Data Set 1 online). The closest homolog of Nb WRKY8 in Arabidopsis is WRKY33 (48.2% amino acid identity).

In tobacco, SIPK, NTF4, WIPK, and NTF6 are characterized as pathogen-responsive MAPKs (Nakagami et al., 2005; Ren et al., 2006). To determine which MAPK phosphorylates WRKY8, we performed in vitro assays using active SIPK, NTF4, WIPK, and NTF6 that had been phosphorylated by cognate recombinant constitutively active MAPKK in vitro. All the active MAPKs showed myelin basic protein (MBP) phosphorylation activity in vitro (see Supplemental Figure 2 online). Thioredoxin (Trx)–fused WRKY8 recombinant protein expressed in Escherichia coli was SIPK-, NTF4-, and WIPK-, but phosphorylation of Trx–WRKY8 by NTF6 was to a lesser degree (Figure 1A). To investigate the phosphorylation site, we prepared the Trx–fused N-terminal half (WRKY81–170) and C-terminal half (WRKY8171–538) of recombinant WRKY8, which then underwent an in vitro phosphorylation assay using SIPK. The WRKY81–170, but not WRKY8171–538, was phosphorylated, suggesting that the N-terminal region is phosphorylated by SIPK (see Supplemental Figure 3 online). Potential phosphorylation sites Ser-62, Ser-67, Ser-79, Ser-86, and Ser-98 were identified in the N-terminal region of WRKY8. We generated various recombinant full-length WRKY8 proteins with Ala substitutions at the potential phosphorylation sites. SIPK could hardly phosphorylate the S62A/S67A/S79A/S86A/S98A quintuple mutant, but all single mutations slightly reduced the level of phosphorylation (Figure 1B). The levels of phosphorylation were proportional to the number of phosphorylation sites (Figure 1B, top panel), indicating that Ser-62, Ser-67, Ser-79, Ser-86, and Ser-98 could be independently phosphorylated.

To determine if WRKY8 is phosphorylated in vivo, we prepared antiphosphopeptide antibodies against peptides, including phospho-Ser-79 (pSer79) or phospho-Ser-86 (pSer86) that are highly conserved among group I WRKY proteins (see Supplemental Figure 1B online). Immunoblot analyses showed that antibodies specifically recognized each phosphopeptide used in this study (Figure 1C). We prepared total proteins from N. benthamiana leaves coexpressing WRKY8-HA-StrepII with INF1, but not with GUS-Tactin chromatography, we conducted immunoblot analyses using anti-HA, anti-pSer79, and anti-pSer86 antibodies. Phosphorylation of Ser-79 and Ser-86 was only detected in leaves expressing WRKY8-HA-StrepII with INF1, but not with GUS.
Figure 1. Phosphorylation of WRKY8 in Vitro and in Vivo.

(A) In vitro phosphorylation of WRKY8 by MAPKs. Purified Trx and Trx-fused WRKY8 were used as substrates for active MAPKs. Proteins were separated by SDS-PAGE, were stained with Coomassie Brilliant Blue (CBB; bottom panel), and were exposed to x-ray film (kinase assay; top panel).

(B) Ala scanning analysis of SIPK-mediated phosphorylation sites in WRKY8. Phosphorylation of WRKY8 variants was detected by autoradiography (middle), and the intensities of each band were quantified (top). Protein loads were monitored by CBB staining (bottom).

(C) Specificity of anti-pSer79 and anti-pSer86 antibodies. Peptides of WRKY874-84, Ser-79–phosphorylated WRKY874-84 (WRKY874-84pSer79) and WRKY881-91, and Ser-86–phosphorylated WRKY881-91 (WRKY881-91pSer86) were spotted on nitrocellulose membranes. Immunoblot analyses were performed using the indicated antibodies.

(D) WRKY8-HA-Strep II FLAG-MEK1/2

(E) WRKY8-HA-Strep II FLAG-MEK1/2

(F) Wrky8-HA-Strep II FLAG-MEK1/2

(G) Wrky8-HA-Strep II FLAG-MEK1/2
(Figure 1D). Immunoblot analysis using anti-HA antibody confirmed similar amounts of WRKY8-HA-StrepII accumulation irrespective of coexpression with INF1 or GUS (Figure 1D). To confirm that phosphorylation of Ser-79 and Ser-86 of WRKY8 is dependent on SIPK, NTF4, and WIPK activities, we analyzed the phosphorylation state of WRKY8-HA-StrepII in response to coexpression of FLAG-MEK2DD or FLAG-MEK2KR (inactive forms of MEK2). Phosphorylation of Ser-79 and Ser-86 was also induced by expression of FLAG-MEK2DD, but not by MEK2KR (Figure 1E). MEK2DD did not directly phosphorylate WRKY8 in vitro, suggesting that WRKY8 is phosphorylated by downstream MAPKs of MEK2 (see Supplemental Figure 4 online).

We evaluated whether phosphorylation of Ser-79 and Ser-86 induced by MEK2DD depends on SIPK, NTF4, or WIPK. We silenced these MAPKs in N. benthamiana using Tobacco rattle virus (TRV)-VIGS constructs (Asai et al., 2008), and WRKY8 phosphorylation was monitored. NTF4 showed high sequence identity to SIPK (Ren et al., 2006), and the VIGS construct of SIPK also silenced NTF4, as also reported by Asai et al. (2008). Because we detected no protein accumulation of WRKY8-HA-StrepII under control of Cauliflower mosaic virus (CaMV) 35S promoter without a p19 silencing suppressor, we used an estradiol-inducible expression system to express WRKY8-HA-StrepII and MEK2DD only in the silenced plants. The estradiol-inducible expression system can control the timing and expression level of the transgene in a dose-dependent manner, and 5 μM estradiol treatment induces the expression of a transgene approximately fourfold higher than a transgene controlled by the CaMV 35S promoter (Zuo et al., 2000). In this study, phosphorylation of Ser-79 and Ser-86 was detected in SIPK/NTF4- or WIPK-silenced leaves in response to FLAG-MEK2DD, but not in SIPK/NTF4/WIPK-silenced leaves (Figure 1F). This result suggests that SIPK/NTF4 and WIPK contribute redundantly to Ser-79 and Ser-86 phosphorylation of WRKY8 in response to MEK2DD expression. We further evaluated the individual contribution of SIPK and NTF4 in WRKY8 phosphorylation. Overexpression of SIPK and NTF4, but not WIPK, show activity by its endogenous upstream kinase-mediated phosphorylation (Zhang and Liu, 2001; Ren et al., 2006). We analyzed the phosphorylation state of WRKY8-HA-StrepII in response to coexpression of FLAG-SIPKWT or FLAG-NTF4WT. Phosphorylation of Ser-79 and Ser-86 was induced by expression of FLAG-SIPKWT and FLAG-NTF4WT, but not by FLAG-SIPKKR and FLAG-NTF4KR (inactive mutants of SIPK and NTF4, respectively) (Figure 1G). These results indicate that overexpression of either SIPK or NTF4 is sufficient to phosphorylate Ser-79 and Ser-86 in plants. The results from these gain-of-function and loss-of-function analyses suggest that SIPK, NTF4, and WIPK are kinases that function upstream of WRKY8.

D Domain–Dependent Interactions Are Required for Phosphorylation of WRKY8 by SIPK

We examined the interaction between WRKY8 and MAPKs by in vitro glutathione S-transferase (GST) pull-down assays. WRKY8-His6 was incubated with glutathione–agarose-bound GST, GST-SIPK, GST-NTF4, GST-WIPK, or GST-NTF6, and the pull-down products were analyzed by immunoblot using anti-His6 antibody. WRKY8-His6 was pulled down with GST-SIPK, GST-NTF4, and GST-WIPK, but not GST and GST-NTF6, suggesting that WRKY8 could interact with SIPK, NTF4, and WIPK (Figure 2A). These results indicate that no other factor is required for interactions between WRKY8 and these MAPKs. Interaction of WRKY8 with SIPK was also examined using a yeast two-hybrid (Y2H) assay. The yeast transformed with full-length WRKY8 that was fused to an activation domain grew on selective plates without specific bait protein. Therefore, the Y2H assay was performed using the N-terminal region of WRKY8 (WRKY81–170), which is sufficient for in vitro phosphorylation by SIPK (see Supplemental Figure 3 online). WRKY81–170 interacted with SIPK, but not with NTF6 (see Supplemental Figure 5 online).

We investigated the subcellular localizations of WRKY8 and MAPKs. WRKY8 and MAPKs were fused to green fluorescent protein (GFP) and were expressed transiently in N. benthamiana leaves by Agrobacterium infiltration (agroinfiltration). Nuclear localization signal–fused GUS (NLS-GUS) was also expressed as a GFP fusion protein, as a positive control of nuclear localization.
Figure 2. D Domain–Dependent Interactions Required for Phosphorylation of WRKY8 by SIPK.

(A) In vitro interaction assays between WRKY8 and MAPKs. GST, GST-SIPK, GST-NTF4, GST-WIPK, and GST-NTF6 purified proteins were incubated with WRKY8-His₆ as indicated. Pulled-down fractions were analyzed by immunoblotting using anti-His₆ antibody (top). Input proteins were monitored by Coomassie blue staining (bottom).

(B) Subcellular localization of WRKY8, SIPK, NTF4, WIPK, and NTF6 in N. benthamiana epidermal cells. N. benthamiana leaves were transformed with GFP-NLS-GUS, GFP-WRKY8, GFP-SIPK, GFP-NTF4, GFP-WIPK, and GFP-NTF6 by agroinfiltration. DAPI, 4’,6-diamidino-2-phenylindole; DIC.

(C) Transient expression of WRKY8WT and WRKY8mD with cYFP and nYFP-SIPK. WRKY8WT and WRKY8mD were co-expressed with cYFP and nYFP-SIPK and subcellular localization of WRKY8 and SIPK were examined by confocal microscopy.

(D) Schematic representation of the WRKY8 and SIPK interaction. The amino acid sequence of WRKY8 and SIPK is shown with the conserved residues highlighted in yellow.

(E) In vitro kinase assays with WRKY8WT and WRKY8mD. WRKY8WT and WRKY8mD were expressed in E. coli and purified using glutathione-Sepharose beads. The purified proteins were incubated with GST-SIPK and the phosphorylated products were analyzed by immunoblotting with anti-His₆ antibody.

(F) Western blot analysis of WRKY8 phosphorylation. WRKY8WT and WRKY8mD were expressed in E. coli and purified using glutathione-Sepharose beads. The purified proteins were incubated with GST-SIPK and the phosphorylated proteins were analyzed by immunoblotting with anti-His₆ antibody.

(G) Time course analysis of WRKY8 phosphorylation. WRKY8WT and WRKY8mD were expressed in E. coli and purified using glutathione-Sepharose beads. The purified proteins were incubated with GST-SIPK for various time periods and the phosphorylated proteins were analyzed by immunoblotting with anti-His₆ antibody.

(H) kinase assay with WRKY8 and SIPK. WRKY8WT, WRKY8mD, and SIPK were expressed in E. coli and purified using glutathione-Sepharose beads. The purified proteins were incubated in a reaction mixture and the phosphorylated products were analyzed by immunoblotting with anti-His₆ antibody.
In vitro analysis of D domain–mutated WRKY8 phosphorylation by SIPK. Purified Trx-fused WRKY8 WT and WRKY8mD were phosphorylated by active SIPK, and WRKY8 was transiently coexpressed in N. benthamiana leaves. No fluorescence was detectable with combinations of cYFP-WRKY8/nYFP-NLS-GUS and cYFP-WRKY8/nYFP-NTF6 (Figure 2C). However, reconstituted YFP fluorescence in the nucleus was detected with combinations of cYFP-WRKY8/nYFP-SIPK, cYFP-WRKY8/nYFP-NTF4, and cYFP-WRKY8/nYFP-WIPK, indicating interactions between WRKY8 and these MAPKs in the nucleus (Figure 2C).

To investigate the contribution of the deduced D domain in the interaction between WRKY8 and SIPK, D domain–mutated WRKY8 (WRKY8mD) and wild-type WRKY8 (WRKY8WT) were used for pull-down, Y2H, and BiFC assays (Figure 2D). In the in vitro pull-down assay, mutations within the D domain disrupted the interaction between WRKY8 and SIPK (Figure 2E). In the Y2H assay, the interaction between WRKY8, 1-179, and SIPK was also reduced by mutation in the D domain (see Supplemental Figure 6 online). In the BiFC assay, WRKY8mD did not interact with SIPK (Figure 2F). These results indicate that the D domain is required for effective interaction between WRKY8 and SIPK. However, interaction between WRKY8 and SIPK did not disappear completely by mutation in the D domain. Therefore, we cannot rule out the possibility that an additional interaction motif for SIPK may exist in WRKY8.

We tested whether the D domain–dependent interaction is required for phosphorylation of WRKY8 by MAPK. We performed in vitro phosphorylation assays using WRKY8WT and WRKY8mD as substrates. The in vitro phosphorylation intensities of WRKY8WT by active SIPK were reduced markedly in WRKY8mD (Figure 2G). Thus, the D domain–dependent interaction is critical for efficient phosphorylation of WRKY8 by SIPK. We also investigated the contribution of the D domain to in vivo phosphorylation of WRKY8 by MAPK. WRKY8WT-HA-Strepll and WRKY8mD-HA-Streplll were coexpressed with either FLAG-MEK2DD or FLAG-MEK2DD in N. benthamiana leaves by agroinfiltration (Figure 2H).

WRKY8 phosphorylation was monitored by immunoblot analyses using anti-pSer79 and anti-pSer86 antibodies. Phosphorylation of Ser-79 and Ser-86 was only detected in leaves expressing FLAG-MEK2DD with WRKY8WT-HA-Strepll, but not with WRKY8mD-HA-Strepll (Figure 2H). These results indicate that D domain–dependent interaction is necessary for MAPK-mediated phosphorylation of WRKY8.

**Phosphorylation of WRKY8 by MAPKs Increases DNA Binding and Transactivation Activities**

WRKY transcription factors mostly likely function by binding to cognate W-box cis-elements in the promoter region of target genes (Eulgem et al., 2000; Ciolkowski et al., 2008). To determine if WRKY8 binds specifically to the W-box, binding of WRKY8 recombinant protein to the W-box was assayed using an electrophoresis mobility shift assay (EMSA). A protein-DNA complex with reduced mobility was detected when recombinant WRKY8 was incubated with the W-box probe, but not with a mutated W-box probe (Figure 3A). The specificity of this binding was also confirmed by competition experiments using an unlabeled probe. These results indicate that WRKY8 specifically binds to the W-box sequence. Phosphorylation affects the DNA binding activity of many transcription factors (Yang et al., 2003). Therefore, we tested if MAPK-mediated phosphorylation of WRKY8 altered its ability to bind to the W-box sequence. Recombinant WRKY8 protein was incubated with wild-type, active, or kinase-inactive SIPK, NTF4, or WIPK and then underwent EMSA. The DNA binding activity of WRKY8 was increased by wild-type SIPK, NTF4, and WIPK (Figure 3B, lanes 4, 6, and 8). By contrast, kinase-inactive SIPK, NTF4, and WIPK did not affect the DNA binding activity (Figure 3B, lanes 3, 5, and 7). These results suggest that MAPK-mediated phosphorylation of WRKY8 promotes its DNA binding activity.

To determine the transactivation activity of WRKY8 in plants, we developed an Agrobacterium-mediated transient assay system. The GUS reporter gene containing an intron to avoid expression in Agrobacterium was fused under the control of a synthetic promoter consisting of the −46 minimal CaMV 3SS promoter and six copies of the GAL4 upstream activation sequence (GAL4UAS) (Figure 3C). The GAL4 DNA binding domain (GAL4DBD) that was fused to the strong activation domain of herpes simplex virus VP16 (GAL4DBD-VP16) strongly activated
Figure 3. Increased DNA Binding and Transactivation Activities by MAPK-Mediated Phosphorylation of WRKY8.

(A) Binding of recombinant WRKY8 to a W-box (W) sequence, but not to a mutated W-box (mW) sequence, using EMSA. Unlabeled W-box fragments and mutated W-box fragments were used as competitor DNAs.

(B) Increased binding activity of WRKY8 to the W-box sequence by MAPK-mediated phosphorylation. After incubation with WRKY8 and SIPKKR (lane 3), SIPKWT (lane 4), NTF4KR (lane 5), NTF4WT (lane 6), WIPKKR (lane 7), WIPKWT (lane 8), or no recombinant MAPK (lane 2), EMSA was done using a 32P-labeled W-box probe. Lane 1 shows mobility of free probe.

(C) Schematic diagram of effector, reporter, and reference plasmids used in transient assays.

(D) High transactivation activity of GAL4DBD-VP16. Data are means ± SD from three independent experiments

(E) Transactivation of the GUS gene by GAL4DBD-WRKY8 variants in N. benthamiana leaves. Total proteins were extracted from leaves coinfiltrated with Agrobacterium-containing reporter plasmids, effector plasmids, or reference plasmids. GUS activity was normalized to LUC activity. AAAAA and DDDDD indicate that WRKY81-170 mutants mimic the nonphosphorylated form and phosphorylated form, respectively. Data are means ± SD from at least three independent experiments. *P < 0.01 versus the GAL4DBD-WRKY81-170 alone by the two-tailed Student’s t test. WT, wild type.

(F) Protein gel blot analysis of GAL4DBD, GAL4DBD-VP16, and GAL4DBD-WRKY81-170 variants. Anti-GAL4 antibody was used to detect accumulation of GAL4DBD and GAL4DBD-fused proteins. Protein loads were monitored by Coomassie Brilliant Blue (CBB) staining of the bands corresponding to ribulose-1,5-bisphosphate carboxylase large subunit (RBCL). Asterisks refer to the pertinent bands.
the GAL4UAS-GUS reporter gene compared with GAL4DBD only (Figure 3D). Because protein accumulation of full-length WRKY8 fused to GAL4DBD could not be detected by immunoblot analyses using anti-GAL4 antibody, WRKY8-1-170 containing an SP cluster was used (Figure 3C). To test the function of phosphorylation of the SP cluster, WRKY8\(^{AAAAA-1-170}\) (mimicking nonphosphorylated WRKY8) and WRKY8\(^{DDDD-1-170}\) (mimicking constitutively phosphorylated WRKY8), in which Ser-62, Ser-67, Ser-79, Ser-86, and Ser-98 are replaced by Ala or Asp, were used. Variants of GAL4DBD-WRKY8-1-170 were expressed in the presence or absence of MEK2\(^{DD}\) and MEK2\(^{DDD}\) in N. benthamiana leaves. The expression of MEK2\(^{DD}\) induces HR-like cell death in tobacco plants (Yang et al., 2001). To synchronize the expression of effector genes and to exclude cell death–associated effects by MEK2\(^{DD}\) on the expression of reporter gene, we used an estradiol-inducible system to make a time lag between the reporter and effector genes after agroinfiltration. At 24 h after agroinfiltration, leaves were injected with estradiol to express MEK2 variants and were incubated for 12 h. Before the onset of visible cell death, the reporter activities were determined. GAL4DBD-WRKY8\(^{WT\-1-170}\) and GAL4DBD-WRKY8\(^{AAAAA-1-170}\) showed no higher GUS activities compared with GAL4DBD (Figure 3E), indicating that WRKY8\(^{WT\-1-170}\) has no transactivation activity. However, we should interpret these results cautiously because WRKY8\(^{WT\-1-170}\) but not full-length WRKY8, was used in these experiments, and accumulation of GAL4DBD-WRKY8 variant proteins was much lower than GAL4DBD (Figure 3F). Coexpression of GAL4DBD-WRKY8\(^{WT\-1-170}\) with MEK2\(^{DD}\), but not with MEK2\(^{DDD}\), increased GUS activity (Figure 3E). By contrast, coexpression of GAL4DBD-WRKY8\(^{AAAAA-1-170}\) with MEK2\(^{DD}\) did not increase GUS activity. GAL4DBD-WRKY8\(^{DDDD-1-170}\) showed massive GUS activities irrespective of MEK2\(^{DD}\). These results indicate that MAPK-dependent phosphorylation of the SP cluster of WRKY8 increases transactivation activity. GAL4DBD-WRKY8\(^{DDDD-1-170}\) showed much higher GUS activity compared with coexpression of GAL4DBD-WRKY8\(^{WT\-1-170}\) and MEK2\(^{DDD}\) (Figure 3E). We think that the difference in GUS activities might be due to dephosphorylation of phosphorylated WRKY8\(^{WT\-1-170}\) by some phosphatases.

MEK2\(^{DD}\) expression induces SIPK, NTF4, and WIPK activation (Ren et al., 2006). To clarify which MAPK-mediated phosphorylation increases transactivation activity of GAL4DBD-WRKY8\(^{WT\-1-170}\), we further analyzed the transactivation activity of GAL4DBD-WRKY8\(^{WT\-1-170}\) in response to SIPK\(^{WT}\) or SIPK\(^{KR}\) expression. Coexpression of GAL4DBD-WRKY8\(^{WT\-1-170}\) with SIPK\(^{WT}\), but not with SIPK\(^{KR}\), increased GUS activity (see Supplemental Figure 7 online), indicating that at least SIPK-mediated phosphorylation increases the transactivation activity of WRKY8.

**Knockdown of WRKY8 Enhances Susceptibility to P. infestans and C. orbiculare**

In this study, the roles of WRKY8 in the basal defense against *P. infestans* and *C. orbiculare*, potent pathogens of *N. benthamiana* (Kamoun et al., 1998; Shen et al., 2001; Takano et al., 2006), were investigated using VIGS based on the TRV vector (Ratcliffe et al., 2001). The oomycete *P. infestans* is a near-obligate pathogen (i.e., a potential biotroph) (Fry, 2008), and the ascomycete fungus *C. orbiculare*, a typical hemibiotroph. The expression level of WRKY8 was markedly reduced in TRV:WRKY8-infected plants compared with the TRV control (Figure 4A). Silencing of WRKY8 appeared to be specific because the mRNA levels of Nb WRKY7, a WRKY closely related to Nb WRKY8 (see Supplemental Figure 1B online), were not affected in *WRKY8*-silenced plants (Figure 4A). Quantitative RT-PCR (qRT-PCR) analyses indicated that TRV:SIPK/WIPK reduced transcript accumulation of SIPK (81.9%), NTF4 (85.4%), and WIPK (82.4%) compared with TRV control plants at 24 h after inoculation with *P. infestans*.

We used qPCR technology to examine changes in *P. infestans* biomass during the interaction with the silenced plants. PCR primers specific to highly repetitive sequences in the *P. infestans* genome were used to quantify relative levels of *P. infestans* DNA in infected plant tissues and were found to reflect an accurate and sensitive estimate of the *P. infestans* biomass (Judelson and Tooley, 2000). Zoospores of a virulent isolate of *P. infestans* were inoculated on the surface of the silenced leaves of *N. benthamiana*. Analysis of *P. infestans* biomass showed that the growth rate of *P. infestans* increased in *WRKY8*-silenced plants compared with TRV-control plants (Figures 4B and 4C). SIPK/NTF4/ WIPK-silenced plants showed a greater decrease in resistance to *P. infestans* compared with WRKY8-silenced plants (Figures 4B and 4C).

*C. orbiculare* conidia were sprayed on the silenced leaves. We counted the number of disease spots, which reflect susceptibility, not HR cell death, on inoculated leaves. The number of disease spots on *WRKY8*-silenced leaves increased compared with that on TRV-control leaves (Figures 4D and 4E). SIPK/NTF4/WIPK-silenced plants developed more disease spots than *WRKY8*-silenced plants (Figures 4D and 4E). These results indicate that *WRKY8* contributes to the defense response against *P. infestans* and *C. orbiculare*, but to a lesser extent than the contribution of SIPK, NTF4, and WIPK, which are the presumed upstream kinases.

**Phosphorylation of WRKY8 Increases Expression of Downstream Defense-Related Genes**

To identify target genes of WRKY8, we used loss-of-function and gain-of-function screening using the suppression subtractive hybridization (SSH) method (Diatchenko et al., 1996). For loss-of-function screening, we constructed an SSH cDNA library from leaves of *WRKY8*-silenced plants expressing MEK2\(^{DD}\) and TRV control plants. Positive clones underwent reverse RNA gel blot analysis for reproducibility and then were analyzed by qRT-PCR. From a total of 1920 randomly selected colonies, we isolated *NADP-malic enzyme (NADP-ME)* as being downregulated in *WRKY8*-silenced plants. NADP-MEs catalyze oxidative decarboxylation of malic acid, producing pyruvate, CO\(_2\), and NADPH, and are suggested to participate in the plant defense response by providing NADPH for defense-related lignification (see Supplemental Figure 8 online) (Wheeler et al., 2005). For gain-of-function screening, we constructed an SSH cDNA library from leaves transformed with the WRKY8\(^{DDDD}\) vector and the empty vector. We used the same procedures as for loss-of-function screening for the analysis. From a total of 960 randomly selected colonies, we isolated 3-hydroxy-3-methylglutaryl CoA reductase.
Increased Disease Susceptibility to a Virulent Strain of *P. infestans* by Silencing of WRKY8.

(A) Specific gene silencing of WRKY8 in TRV:WRKY8-infected plants. Silencing of WRKY8 was monitored by qRT-PCR using specific primers. WRKY7, which shows high homology to WRKY8 (see Supplemental Figure 1B online), was used as a silencing control. Data are means ± SD from three independent experiments.

(B) Susceptibility to *P. infestans* in silenced plants. Photographs were taken 5 d after the inoculation.

(C) Effects of infection of TRV:WRKY8 (8) or TRV:SIPK/WIPK (S/W) on *P. infestans* infection. Data are means ± SD from three independent experiments.

(D) Susceptibility to *C. orbiculare* in silenced plants. Photographs were taken 5 d after the inoculation.

(E) Effects of infection of TRV:8 or TRV:S/W on *C. orbiculare* infection. The number of disease spots in the leaves was counted 5 d after the inoculation. Data are means ± SD from four independent experiments. *P < 0.05 and **P < 0.01; two-tailed Student’s *t* test.

2 (HMGR2) as an upregulated gene from leaves expressing WRKY8DDDDDD, HMGR catalyzes the rate-limiting step in the biosynthesis of all isoprenoid compounds, including capsidiol, the major tobacco phytoalexin accumulated during the defense response (see Supplemental Figure 8 online) (Wu et al., 2006). qRT-PCR was used to validate that these genes (*NADP-ME* and *HMGR2*) function downstream of WRKY8. The expression levels of *NADP-ME* and *HMGR2* were significantly upregulated in response to MEK2DD expression and *P. infestans* infection (Figure 5A). To evaluate the contribution of WRKY8 and SIPK/NFT4/WIPK to the upregulation of these genes, we used the TRV-based VIGS method to silence the target genes. We detected no significant induction of *NADP-ME* and *HMGR2* genes after TRV infection (see Supplemental Figure 9 online). The upregulation of *NADP-ME* and *HMGR2* in response to MEK2DD expression and *P. infestans* infection was compromised in WRKY8-silenced plants (Figure 5A). The expression levels of both genes decreased more in SIPK/NFT4/WIPK-silenced plants (Figure 5A). These results indicate that accumulation of *NADP-ME* and *HMGR2* mRNAs depends on SIPK, NFT4, and WIPK activation and requires WRKY8. Our data support the previous report by Zhang and Liu (2001) describing that ectopic expression of SIPK induces HMGR in *N. tabacum*.

To examine the effect of WRKY8 phosphorylation on the induction of *NADP-ME* and *HMGR2*, we expressed HA-tagged WRKY8 (WRKY8WT) and its mutants, WRKY8AAAAA and WRKY8DDDDDD, in *N. benthamiana* leaves, and we analyzed the expression levels of both genes. WRKY8AAAAA and WRKY8WT induced expression of *NADP-ME* and *HMGR2* to some extent (Figure 5B). In a transactivation assay, GAL4DBD-WRKY8WT1-170 and GAL4DBD-WRKY8AAAAA1-170 showed no higher GUS activities compared with GAL4DBD (Figure 3E). However, accumulation of GAL4DBD-WRKY8 variant proteins was much lower than GAL4DBD (Figure 3F). We think that the nonphosphorylated form of WRKY8 has basal transactivation activity because WRKY8AAAA bound to the W-box sequence (Figure 3A). WRKY8DDDDDD highly induced the expression of both genes compared with WRKY8AAAAA and WRKY8WT (Figure 5B), indicating that the phosho-mimicking mutation of WRKY8 results in the induction of downstream genes. We made expression analyses of *NADP-ME*, *HMGR2*, and WRKY8 in response to syringe infiltration of water or to INF1 elicitor, which activates prolonged MBP kinase activities in kinases with a molecular mass of 48 and 44 kD (see Supplemental Figure 10A online). SIPK and NFT4 are 48 kD, and WIPK is 44 kD (Ren et al., 2008), 48-kD MBP kinases are presumed to correspond to 44-kD MBPs kinase to WIPK. INF1 elicitation treatment may induce activation of SIPK, NFT4, and WIPK. Although no obvious difference was observed in the accumulation profile of WRKY8 mRNA between water treatment and INF1, accumulation of *NADP-ME* and *HMGR2* transcripts increased in response to INF1 treatment (see Supplemental Figure 10B online), suggesting that upregulation of WRKY8 mRNA is not enough to induce expression of *NADP-ME* and *HMGR2*. These results together suggest that MAPK-dependent phosphorylation
of WRKY8 positively regulates expression of downstream genes NADP-ME and HMGR2.

DISCUSSION

MAPK-Mediated Phosphorylation of WRKY8 Regulates Defense Responses as a Positive Transcription Factor

MAPKs have pivotal roles in induced defense responses of plants. However, the regulatory mechanisms by which MAPKs induce defense responses are unclear. In this study, we showed that WRKY8 is phosphorylated by SIPK, NTF4, and WIPK in vivo and that D domain–dependent interaction is necessary for SIPK-mediated phosphorylation of WRKY8. We also showed that MAPK-mediated phosphorylation of WRKY8 activates its DNA binding and transactivation activities and that WRKY8 induces downstream defense genes (Figure 6). D domain–mutated WRKY8 was not phosphorylated in response to MEK2DD expression, which induces SIPK, NTF4, and WIPK activation (Figure 2H). Because NTF4 and WIPK interacted with and phosphorylated WRKY8 in the same way as SIPK (Figures 1 and 2), we hypothesize that phosphorylation of WRKY8 by NTF4 and WIPK also requires D domain–dependent interaction (Figure 6).

One major mechanism to effect changes in gene expression appears to be through MAPKs altering the activity of transcription factors and hence transcription of their cognate target genes (Yang et al., 2003). Phosphorylation of transcription factors by MAPK can alter (1) cellular localization, (2) protein stability, (3) DNA binding activity, (4) transactivation or repression activity, and (5) remodeling of nucleosome structure (Yang et al., 2003; Tootle and Rebay, 2005). We showed that SIPK can phosphorylate Ser residues within the SP cluster and that SIPK-mediated phosphorylation of WRKY8 increases both DNA binding and transcriptional activities (Figures 1 and 3; see Supplemental Figure 7 online). Ectopic expression of the SP cluster phosphomimicking mutant WRKY8DDDD induced downstream target genes NADP-ME and HMGR2 (Figure 5B). These results support the idea that phosphorylation of WRKY8 by SIPK functions in (3) and (4) above. Even though we could not indicate individual functions of MAPKs for WRKY8, silencing of WIPK is required to eliminate SP cluster phosphorylation of Ser-79 and Ser-86 by MEK2DD (Figure 1F), and overexpression of SIPK or NTF4 induced Ser-79 and Ser-86 phosphorylation (Figure 1G). NTF4- or WIPK-mediated phosphorylation of WRKY8 also promoted its DNA binding activity (Figure 3B). GAL4DBD-WRKY8DDDD showed massive transactivation activity irrespective of MEK2DD (Figure 3E). These results suggest that SIPK, NTF4, and WIPK may phosphorylate similar sites and have roles similar to WRKY8 in (3) and (4) above. In mammals, Elk-1 transcription factor, a substrate of MAPKs, increases both DNA binding and transactivation

Figure 5. Induction of Target Gene Expression by a Phosphorylation-Mimicking Mutant of WRKY8.

(A) Expression levels of NADP-ME (top) and HMGR2 (bottom) induced by MEK2DD (left) or P. infestans (right) were compromised by TRV:WRKY8 or TRV:SIPK/WIPK. Data are means ± SD from three independent experiments.

(B) Expression of NADP-ME and HMGR2 in response to WRKY8 variants. Total RNAs were extracted from N. benthamiana leaves 48 h after agroinfiltration and were used for qRT-PCR. Data are means ± SD from at least three independent experiments. Anti-HA antibody was used to detect accumulation of WRKY8-HA variants. Protein loads were monitored by CBB staining of the bands corresponding to RBCL. WT, wild type.

*P < 0.05 and **P < 0.01; two-tailed Student’s t test.

[See online article for color version of this figure.]
activities that depend on phosphorylation at multisite residues within the transcriptional activation domain (TAD) (Yang et al., 1999, 2003; Tootle and Rebay, 2005). The TAD of Elk-1 binds directly to the ETS domain, which is a helix-turn-helix DNA binding domain, limiting interaction with DNA until the TAD is phosphorylated (Yang et al., 1999). In this case, phosphorylation by MAPKs triggers release of autoinhibition in Elk-1. Future studies will examine if WRKY8 is regulated in a way similar to Elk-1.

In this study, silencing of WRKY8 decreased expression levels of NADP-ME and of HMGR2 induced by MEK2DD expression and P. infestans infection (Figure 5A). WRKY8-silenced plants showed increased disease susceptibility to the potential biotroph P. infestans and the hemibiotroph C. orbiculare. These results indicate that WRKY8 regulates broad-spectrum disease resistance through activation of target defense genes. However, the decrease in the transcript level of both genes by WRKY8 silencing was much less than by SIPK/NTF4/WIPK silencing (Figure 5A). Similarly, SIPK/NTF4/WIPK-silenced plants showed a greater increase in susceptibility than WRKY8-silenced plants (Figure 4). Redundancy of genes encoding plant transcription factors complicates loss-of-function analyses that aim to identify the biological functions of large families that contain members with strongly conserved DNA binding domains (Mitsuda and Ohme-Takagi, 2009). Structurally closely related Arabidopsis WRKY11 and WRKY17 act as negative regulators of basal resistance to bacterial pathogens (Journot-Catalino et al., 2006). Expression analysis of genes regulated by WRKY11 or WRKY17 or both showed that WRKY11 and WRKY17 act partially redundantly to a subset of target genes (Journot-Catalino et al., 2006). These observations suggest that WRKY8 functions redundantly with other group I WRKYs that are not silenced by TRV:WRKY8 because of mismatching of its nucleotide sequence and that silenced plants show weak phenotypes. Alternatively, because MAPKs are potentially capable of phosphorylating various transcription factors in vitro (Popescu et al., 2009), such factors could also regulate target genes directly or indirectly.

The SP Cluster of the N-Terminal Region of WRKY Is a Target Sequence of MAPK

WRKY8 was phosphorylated by SIPK, NTF4, and WIPK (Figures 1 and 2). Other studies showed that Arabidopsis MPK3 and MPK6 (WIPK and SIPK orthologs, respectively) have a high level of functional redundancy (Pitzschke et al., 2009), suggesting that MPK3 and MPK6 might share some of their substrates. ETHYLENE INSENSITIVE3, PHOS32, and SPEECHLESS are substrates of both MPK3 and MPK6 (Lampard et al., 2008; Merkouropoulos et al., 2008; Yoo et al., 2008). Extensive in vitro analysis showed that MPK6 shares 40% of in vitro substrates with MPK3 (Popescu et al., 2009). In this study, we showed that WRKY8 is a target of SIPK, NTF4, and WIPK and that WRKY8 appears to be phosphorylated within the SP cluster, is activated by these MAPKs, and participates in defense-related signaling pathways. To our surprise, the SP cluster is highly conserved in the N-terminal region of several group I WRKYs, including Arabidopsis WRKY25 and WRKY33 (see Supplemental Figure 1B online), which are phosphorylated by MPK4 in vitro (Andreasson et al., 2005), and Nt WRKY1, which is phosphorylated by SIPK in vitro (Menke et al., 2005). Eulgem and Somssich (2007) described the possibility that the D-motif, a conserved pattern of SP dimers within group I WRKYs, is a consensus phosphoacceptor site for MAPKs. Because Ser-79 and Ser-86 of WRKY8 correspond to SP dimers within the D motif, our study provides evidence that the D-motif can be a MAPK target sequence. The Ala scanning analysis indicated that adjacent SP sequences could be phosphorylated by MAPKs (Figure 1B). We propose that WRKYs containing an SP cluster might be a substrate of certain MAPKs. Although the phosphorylation site of Nt WRKY1 by SIPK was not identified, SIPK-mediated phosphorylation of Nt WRKY1 increased its DNA binding activity in vitro (Menke et al., 2005). Transient coexpression of Nt WRKY1 with SIPK indicated that Nt WRKY1 positively participates in SIPK-dependent cell death (Menke et al., 2005). We hypothesize that the activation method of the SP cluster of Nt WRKY1 might be similar to that of Nb WRKY8.

Some SP cluster-containing WRKYs have a D domain–like sequence (see Supplemental Figure 1B online). In this study, we provided biochemical evidence that Nb WRKY8 directly interacts with SIPK, NTF4, and WIPK and that interaction between Nb WRKY8 and SIPK requires a D domain (Figure 2). Because the phosphorylation intensity of the D-domain–mutated Nb WRKY8

![Figure 6. Model of the Regulatory Mechanism of WRKY8 by MAPK-Dependent Phosphorylation.](Image)

Pathogen recognition leads to activation of MEK2 by unidentified MAP3K (s). Active MEK2 phosphorylates and activates SIPK, NTF4, and WIPK. Active SIPK interacts with WRKY8 in a D domain–dependent manner and phosphorylates WRKY8. Active NTF4 and WIPK may act in a way similar to SIPK. Phosphorylated and activated WRKY8 binds to W-box sites within its target genes and upregulates the expression of these genes.

Some SP cluster–containing WRKYs have a D domain–like sequence (see Supplemental Figure 1B online). In this study, we provided biochemical evidence that Nb WRKY8 directly interacts with SIPK, NTF4, and WIPK and that interaction between Nb WRKY8 and SIPK requires a D domain (Figure 2). Because the phosphorylation intensity of the D-domain–mutated Nb WRKY8
MAPKs Have Dual Functions in Phosphorylation-Dependent Modulation and Sequestration of the Transcription Factor

Bacterial PAMP flg22 and pathogens cause dissociation of an MPK4/MKS1/WRKY33 complex in Arabidopsis (Qiu et al., 2008). Phosphorylation of MKS1 by activated MPK4 triggers the release of MKS1 and WRKY33; thus, WRKY33 can activate the expression of target genes containing PHYTOALEXIN DEFICIENT3 (PAD3), which encodes cytochrome P450 monooxygenase and is required for the last step of the synthesis of camalexin. In this situation, MPK4 indirectly interacts with WRKY33. No interaction has been detected between WRKY33 and MPK4 by Y2H assay (Andreason et al., 2005). By contrast, in this study, Nb WRKY8 directly interacts with SIPK, NTF4, and WIPK, and the interaction between WRKY8 and SIPK requires a D domain (Figure 2). Our study suggests that both the D domain– and the SP cluster–containing WRKY proteins, such as WRKY33, might be phosphorylated by certain MAPKs and positively regulate defense responses. The MPK3/MPK6 cascade regulates camalexin synthesis through transcriptional activation of the PAD3 gene, which is the downstream target of WRKY33 (Ren et al., 2008). We hypothesize that unassociated WRKY33 may be recognized by MPK3/MPK6 directly after WRKY33 is induced by pathogen signals (Lippok et al., 2007), possibly because the stoichiometric imbalance resulting from the synthesis of WRKY33 does not allow the recruitment of WRKY33 into the MKS1 complex. Expression of WRKY8 was also upregulated by pathogen attack and mechanical stress (Figure 4A; see Supplemental Figure 10B online), suggesting that WRKY8 may exist in an unassociated form, even although a counterpart of MKS1 is unknown from online), suggesting that WRKY8 may exist in an unassociated and mechanical stress (Figure 4A; see Supplemental Figure 10B online). Because the most commonly used protease in proteome analysis is trypsin, which cleaves the C-terminal to Arg and Lys residues, characterizing a protein region with very few or even with no such basic residues is difficult. Although we made a mass analysis of trypsin-digested WRKY8 that was phosphorylated by MAPK in vitro, using liquid chromatography–tandem mass spectrometry, fragments corresponding to the SP cluster were not detected.

WRKYs Containing the SP Cluster Participate in Diverse Biological Processes in Plants

MAPKs regulate a diverse set of processes, including abscission, stomatal and ovule development, signals for various abiotic stresses, and defense responses against bacterial and fungal pathogens (Pitzschke et al., 2009; Rodriguez et al., 2010). At WRKY2, which contains the SP cluster, has been implicated in seed germination and postgermination arrest of development by abscisic acid, which activates MPK4 and MPK6 (Xing et al., 2008; Jiang and Yu, 2009). Likewise, At WRKY10 is expressed in developing endosperm and participates in seed development (Luo et al., 2005). Overexpression of rice WRKY53, which is the closest homolog of Nb WRKY8 in rice, upregulates several defense-related genes in rice cells (Chujo et al., 2007). Nt WRKY1 appears to participate in the initiation of HR-like cell death (Menke et al., 2005). These studies suggest that WRKYs containing an SP cluster may contribute to the regulation of many biological processes; thus, MAPK-mediated phosphorylation within the SP cluster may participate in responses other than defense responses. Currently, we do not know how certain MAPKs regulate a wide variety of events in response to biotic stimuli or in plant growth. Temporal and spatial expression profiles of group I WRKYs, in particular cell types at particular developmental stages or under particular environmental conditions, and comprehensive biochemical analyses will provide insight into the detailed mechanisms underlying the pleiotropic functions of MAPK.

METHODS

Plant Growth Conditions

Nicotiana benthamiana plants were grown at 25°C and 70% humidity under a 16-h photoperiod and an 8-h dark period in environmentally controlled growth cabinets.

Pathogen Inoculation

Phytophthora infestans zoospore inoculation was done as described by Yoshioka et al. (2003). For expression analysis, leaves were inoculated with P. infestans race 1.2.3.4 zoospore suspension (1 × 10⁶ zoospores/mL).
For pathogen growth assays, leaves were inoculated with *P. infestans* race 1.2.3.4 zoospore suspension (1 × 10^4 zoospores/mL). Determination of *P. infestans* biomass was done as described by Asai et al. (2010). *Colletotrichum orbiculare* strain 104-T conidia suspension was prepared and inoculated as described by Asai et al. (2008).

**Plasmid Constructs for Recombinant Proteins**

PCR fragments of WRKY8 were inserted into the MscI/HindIII sites of the pET-32a vector (Novagen). The following primer sets were used: WRKY8 (5'-TTGGGCGCATATGGCGAGATCTCTCACACTAG-3' and 5'-CCGCTGACATGGCAGCTGCTTTCTTGATTCTTG-3'), and WRKY8<sub>G71,338</sub> (5'-TTCTG-GGACATCTGCCAATGACATCAATC-3' and 5'-CCGCTGACATGGCAGCTGCTTTCTTGATTCTTG-3') and 5'-CCCAGCTTGGCCATATGGCAGCTTCTTCAACAATCATAG-3'). The PCR fragments of Nb SIPK, Nb NTF4, and Nb WIPK were inserted into the SpeI/BamHI sites of the pET-32a vector (Novagen). The following primers were used: Nb SIPK (5'-GGACTAGTATGGATGACGCTCTGGTGGG-3', Nb MEK1DD (5'-GGACTAGTATGGCTGATGCAAATATGGG-3', Nb WRKY8 (5'-GGACTAGTATGGAAAACGAAACCAATGA-3'), Nb SIPK (5'-GGACTAGTATGGCTGATGCAAATATGGG-3', and Nb WIPK (5'-GGGATCCTTAAGCATATTCAGGATTCAGCG-3'). Amino acid substitutions were introduced by PCR-based, site-directed mutagenesis using a PrimeSTAR mutagenesis basal kit (Takara Bio). The PCR fragment of Nb MEK1DD, which was amplified from plasmids containing full-length cDNA as a template, was inserted into the SacI/Xhol site of the pET-32a vector. The following primer was used: Nb MEK1DD (5'-CCGAGCTCATGGAAGAAGGCAAGCTATGCAATCATAGAATCTGTTGATCAG-3'). The resulting fragments were puriﬁed by denaturing gel electrophoresis and desalted before transformation into *E. coli* BL21-CodonPlus (DE3)-RIPL (Stratagene). The overnight culture at 37°C was transferred to 100-fold LB medium containing 50 μg/mL kanamycin, and then incubated to OD<sub>600</sub> 1.0 at 37°C. Protein synthesis was induced by adding 0.2 mM IPTG for 4 h at 23°C. Each constitutively active MAPKK was extracted and puriﬁed using Ni Sepharose 6 Fast Flow according to the manufacturer’s instructions.

**Preparation of Active MAPKs**

In vitro phosphorylation of wild-type or kinase-inactive Nb SIPK, Nb NTF4, and Nb WIPK by Nb MEK1DD, and Nb NTF6 by Nb MEK1DD, was performed in a phosphorylation buffer (20 mM HEPES-KOH, pH 7.6, 10 mM MgCl<sub>2</sub>, and 1 mM DTT) containing 100 mM Na<sub>2</sub>VO<sub>4</sub>, 5 mM NaF, 5 mM β-glycerophosphate, 500 μM ATP, 0.3 mg MAPK, and 0.075 mg constitutively active MAPKK at 30°C for 30 min. To remove MAPKK, S-protein-agarose (Novagen) equilibrated with phosphorylation buffer was added. After 30 min incubation at room temperature with gentle shaking, the agaro-se-protein complex was removed by brief centrifugation. The resulting supernatants containing active forms of MAPKs were concentrated using Microcon 10 (Millipore) and were stored at −80°C in phosphorylation buffer containing 50% glycerol.

**In Vitro Phosphorylation**

Recombinant WRKY8 protein (0.025 μg/mL) or MBP (0.1 μg/mL; Sigma-Aldrich), which was incubated with 0.01 μg/mL of active MAPKs in phosphorylation buffer (20 mM HEPES-KOH, pH 7.6, 1 mM DTT, and 10 mM MgCl<sub>2</sub>) containing 50 μM ATP and 50 μCi/mL [γ<sup>32</sup>P]ATP at 30°C for 30 min. The reactions were stopped by adding 2× SDS-PAGE sample buffer. Kinase activities were analyzed by SDS-PAGE followed by autoradiography.

**Antibody Production and Immunoblotting**

The peptides for pSer-79 (CIPPGPSLPO<sub>H</sub>TPTELL) and pSer-86 (CETELDDSPO<sub>H</sub>HPILLLS) were synthesized and were conjugated to keyhole limpet hemocyanin carrier, and the polyclonal antisera were raised in rabbits (BioGate). The pSer-79–specific antibody and pSer-86–specific antibody were purified by affinity chromatography using pSer-79 and pSer-86 peptide coupled to Hitrap NHS-activated columns (GE Healthcare). The eluates were then passed through an affinity column coupled to a nonphosphorylated peptide. FLAG- and HA-tagged proteins were detected by monoclonal anti-FLAG antibody (clone M2; Sigma-Aldrich) and monoclonal anti-HA antibody (clone HA-7; Sigma-Aldrich), respectively. Fusion proteins of GAL4 DBD were detected by monoclonal anti-GAL4 antibody (clone RK51; Santa Cruz).

For immunoblotting, equal amounts of proteins were separated on an SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane (Whatman). After blocking in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk or 3% BSA for 1 h at room temperature, the membranes were incubated with anti-pSer79, anti-pS86, anti-FLAG, anti-GAL4, or anti-HA antibodies diluted with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). After washing with TBS-T, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit Ig or anti-mouse Ig antibody (GE Healthcare) for 1 h at room temperature. The antibody-antigen complex was detected using an enhanced chemiluminescence detection kit (Amersham). The membranes were exposed to X-ray film for 20–30 min, and color was developed using X-ray Developer (Amersham). The bands were quantitated by using a digital imaging system (UVP, Cambridge, MA).

**Preparation of Protein Extracts and Strep-Tactin Affinity Chromatography**

Leaves were ground in liquid nitrogen with a mortar and pestle and were thawed in lysis buffer (20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, 1% Triton X-100, 40 mM β-glycerophosphate,
10 mM NaF, 1 mM Na₂VO₄, 5 mM DTT, and 1% Protease inhibitor cocktail for plant cell tissue extracts [Sigma-Aldrich]). The homogenate was incubated at 4°C for 10 min, and then 5 M NaCl was added to a final concentration of 420 mM. The mixture was incubated at 4°C for 1 h with occasional mixing and was then sonicated to reduce viscosity. The mixture was centrifuged at 20,000g for 10 min at 4°C, and the supernatant was used for Strep-Tactin affinity chromatography of StrepII-tagged proteins. After adjusting the protein concentrations, the protein extracts (600 μL) were diluted with 1 mL of dilution buffer (20 mM HEPES-NaOH, pH 7.5, 5 mM DTT, 10% glycerol, 25 μg/mL avidin, and 1% Triton X-100). Forty microliters of MagStrep type 2 Beads (IBA), which were preequilibrated with wash buffer (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 5 mM DTT, 10% glycerol, and 1% Triton X-100), were added, and the mixture was incubated overnight at 4°C with gentle shaking. The resin was washed three times with wash buffer and finally was resuspended in 30 μL of 2× SDS-PAGE sample buffer. The protein concentration was determined using the Protein Assay Dye Reagent (Bio-Rad Laboratories) with BSA as a standard.

**GFP Fusions and BiFC Constructs for Expression in N. benthamiana**

For subcellular localization analysis, cDNA fragments of Nb WRKY8, Nb SIPK, Nb NTF4, Nb WIPK, Nb NTF6, and NLS-GUS were cloned into pGWBI06, which fused GFP to the N terminus of the protein [Nakagawa et al., 2007]. Each plasmid was transformed into Agrobacterium tumefaciens GV3101. Infiltration of Agrobacterium was done at OD₆₀₀ 0.5. For BiFC studies, a cDNA fragment of full-length Nb WRKY8 was cloned into a binary vector, which fused the N-terminal half of YFP (nYFP) to the N terminus of the protein. cDNA fragments of Nb SIPK, Nb WIPK, Nb NTF6, and NLS-GUS were cloned into a pGWBI-based binary vector, which fused the C-terminal half of YFP (cYFP) to the N terminus of the protein. Binary plasmids were transformed into Agrobacterium GV3101. Confrontation of Agrobacterium containing the BiFC constructs was done at OD₆₀₀ 0.25:0.25.

**Microscopy**

Epidermal cell layers of N. benthamiana leaves were assayed for fluorescence 48 h after agroinfiltration. Cells were visualized using a fluorescent microscope (Axio Imager M1; Carl Zeiss) with fluorescent filters: filter set 38E (excitation BP 470/40, beam splitter FT 395, emission BP 525/50) for GFP and YFP and filter set 49 (excitation G 365, beam splitter 475/50) for GFP and YFP and filter set 43 (emission 48 h) for 4’-6-diamidino-2-phenylindole. Images were collected using a CCD camera (AxioCam HRc; Carl Zeiss).

**In Vitro Pull-Down Assay**

For pull-down assays, 3 μg of N-terminal GAL4 upstream activation domain (5’-CGGAGTACTGTCCTCG-3’) was fused upstream of the minimal 35S promoter (~46) and sequence and were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the minimal 35S promoter (~46) and sequence and were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter.

**EMSAs**

Double-stranded synthetic oligonucleotides were end-labeled with 32p by Megalabel (Takara Bio). The probe was purified using a MicroSpin G-50 column (GE Healthcare). Phosphorylation of WRKY8 recombinant proteins was described in the section entitled Expression and Purification of Recombinant Proteins. The EMSA reaction mixture comprised 25 mM HEPES-KOH, pH 7.6, 40 mM KCl, 1 mM DTT, 10% glycerol, 5 μg poly (dl-dC) (GE Healthcare), 0.4 μg recombinant WRKY8, and 1 μL (600,000 cpm/μL) of the probe in a final volume of 16 μL. DNA-protein complexes were allowed to form for 20 min at 30°C and were separated on a 10% polyacrylamide gel in 0.5× TBE at 4°C. Bands were visualized by autoradiography.

**Assays of Transactivation Activity**

For reporter plasmids, six tandem repeats of the GAL4 upstream activation sequence (5’-CGGAGTACTGTCCTCG-3’) was fused upstream of the minimal 35S promoter (~46) and sequence and were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was subsequently cloned behind the synthetic promoter. For effector plasmids, the N-terminal cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into pGreen.

**Y2H Assay**

cDNA fragments of WRKY8 WT or its derivatives plasmaid were cloned into the pGADT7 vector in-frame with the GAL4 activation domain. cDNA fragments of Nb SIPK and Nb NTF4 were cloned into the pGBKKT7 vector in-frame proximal to the binding domain. These vectors were cotransformed into yeast AH109 (Clontech) using the protocol Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Cotransformants were selected on SD-LW (synthetic dropout medium lacking Leu and Trp). The interactions were tested on selective medium (SD-LWAH) lacking Leu, Trp, adenine, and His, according to the manufacturer’s instructions. Serial 1:10 dilutions were prepared in water, and 4 μL of each dilution was used to yield one spot. Plates were incubated at 30°C for 2 d before scoring and taking photographs. SV40 T-antigen with p53 or Lamin C was used as the positive and negative control, respectively.

**VIGS**

VIGS was performed as previously described by Ratcliffe et al. (2001). The following primers were used to amplify cDNA fragments of WRKY8 from an N. benthamiana cDNA library [Yoshioka et al., 2003], and restriction sites were added to the 5’-ends of the forward and reverse primers for cloning into the TRV vector pTV00 (RNA2); WRKY8-F-SalHI

yeast AH109 (Clontech) using the protocol Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Cotransformants were selected on SD-LW (synthetic dropout medium lacking Leu and Trp). The interactions were tested on selective medium (SD-LWAH) lacking Leu, Trp, adenine, and His, according to the manufacturer’s instructions. Serial 1:10 dilutions were prepared in water, and 4 μL of each dilution was used to yield one spot. Plates were incubated at 30°C for 2 d before scoring and taking photographs. SV40 T-antigen with p53 or Lamin C was used as the positive and negative control, respectively.
cDNA fragments of Nt at 23 by viruses by means of Agrobacterium were described by Tanaka et al. (2009). N. benthamiana was transfected by viruses of means of Agrobacterium-mediated transient expression of infectious constructs. The vectors pBINTRAP and pTV00, containing the inserts RNA1 and RNA2, respectively, were transformed separately by electroporation into Agrobacterium GV3101, which includes the transformation helper plasmid pSoup (Hellens et al., 2000). A mixture of equal parts of Agrobacterium suspensions containing RNA1 and RNA2 was inoculated into 2- to 3-week-old N. benthamiana seedlings. The inoculated plants were grown under a 16-h photoperiod and an 8-h dark period at 23°C. The upper leaves of the inoculated plants were used for assays 3 to 4 weeks after inoculation.

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cDNA fragments of Nt MEK2 (5'-CGGGATCATCTTATTTTCTTTTAATTCC-3') and WRKY8-R-CIal(5'-CATCAGATGAGATCAGTGAATGAGAAACTGTG-3'). Amplification using these primers produced a 180-bp fragment, pTVo0 vectors containing the cDNA fragments of Nb SIPK, Nb WIPK, or Nb SIPK/Nb WIPK were described by Tanaka et al. (2009). N. benthamiana was transfected by viruses of means of Agrobacterium-mediated transient expression of infectious constructs. The vectors pBINTRAP and pTV00, containing the inserts RNA1 and RNA2, respectively, were transformed separately by electroporation into Agrobacterium GV3101, which includes the transformation helper plasmid pSoup (Hellens et al., 2000). A mixture of equal parts of Agrobacterium suspensions containing RNA1 and RNA2 was inoculated into 2- to 3-week-old N. benthamiana seedlings. The inoculated plants were grown under a 16-h photoperiod and an 8-h dark period at 23°C. The upper leaves of the inoculated plants were used for assays 3 to 4 weeks after inoculation.

For loss-of-function screening, total RNA was extracted from leaves expressing WRKY8DDDDD or the empty vector (pER8) at 3 h after 17-methyl estradiol treatment. Poly(A)+ RNA was purified using Oligotex-dt30 (Takara Bio). In-gel kinase assays were performed as described by Katou et al. (1999). Briefly, 20 mg of N. benthamiana soluble proteins were separated on a 10% SDS-polyacrylamide gel in the presence of 0.25 mg/mL MBP. After electrophoresis, SDS was removed by washing the gel in 20 mM Tris-Cl, pH 8.0, and 20% 2-propanol four times for 30 min each. The gel was washed in buffer A (20 mM Tris-Cl, pH 8.0, and 1 mM DTT) twice for 30 min each and then was denatured in buffer A containing 6 M guanidine hydrochloride twice for 30 min each. The proteins were renatured overnight at 4°C by incubating the gel in buffer A containing 0.03% Tween 20 with four changes of the solution. The gel was equilibrated in 20 mM HEPES-KOH, pH 7.6, 10 mM MgCl2, and 1 mM DTT and then incubated in the same buffer containing 25 μM ATP and 0.5 μCi/mL [γ-32P]ATP (4000 Ci/mmol) for 1 h at room temperature. The reaction was stopped by washing the gel in 5% trichloroacetic acid and 1% sodium pyrophosphate. The gel was washed extensively with this solution and was washed in 20% methanol, dried under vacuum, and autoradiographed.

**Phylogenetic Analysis**

For the phylogenetic analysis, the program MEGA 4.1 (Tamura et al., 2007) was used. The protein sequences aligned using the ClustalW method in MEGA 4.1 (pairwise alignment: gap opening penalty 10, gap extension penalty 0.1; multiple alignment: gap opening penalty 0.2, protein weight matrix using Gonnet). The residue-specific and hydrophilic penalties were ON, whereas the End Gap separation and the Use negative separation were OFF. Gap separation distance used was 4, and the delay divergence cutoff was at 30. This alignment (available as Supplemental Data Set 1 online) was then used for a bootstrap test (3000 replicates, seed = 35,342) for phylogeny using the neighbor-joining method (p-distance model, pairwise deletion of gaps).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL/DBJ data libraries under accession numbers AB555919 (Nb NADP-ME), AB555290 (Nb HMGR2), AB445391 (Nb WRKY7), AB445392 (Nb WRKY8), AB373026 (Nb NAD), AB360634 (Nb NTF4), AB373025 (Nb SIPK), AB098729 (Nb WIPK), AB360635 (Nb MEK1), and AB091780 (St MEK2). Accession numbers of the amino acid sequences included in the phylogenetic tree are presented in Supplemental Table 1 online.
Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure 1. SP Cluster and D Domain Widely Conserved in the N-Terminal Region of Group I WRKY Proteins.
Supplemental Figure 2. In Vitro Phosphorylation of MBP by Recombinant MAPKs.
Supplemental Figure 3. The N-Terminal Region of WRKY8 Is Phosphorylated by SIPK.
Supplemental Figure 4. In Vitro Phosphorylation Analysis of WRKY8 by Recombinant MEK2DD.
Supplemental Figure 5. Y2H Analysis of the Interaction between WRKY81-170 and SIPK.
Supplemental Figure 6. Y2H Analysis of the Interaction between WRKY81-170 and SIPK.
Supplemental Figure 7. Increased Transactivation Activity of GAL4DBD-WRKY8WT1-170 by SIPK-Mediated Phosphorylation.
Supplemental Figure 8. Schematic Representation of Defense-Related Metabolic Pathways Induced by WRKY8.
Supplemental Figure 9. Expression Analysis of NADP-ME and HMGR2 in N. benthamiana in Response to TRV Infection.
Supplemental Figure 10. Activation of MBP Kinase Activity and Expression of Downstream Genes of WRKY8 Induced by INF1 Treatment.
Supplemental Table 1. Accession Numbers of the Amino Acid Sequences Included in the Phylogenetic Tree.
Supplemental Data Set 1. Text File of Alignment Used to Generate Figure 1B.

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